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Induction of interleukin-6 in human retinal epithelial cells by an attenuated Herpes simplex virus vector requires viral replication and NFκB activation

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Abstract

Gene delivery has potential for treating ocular disease and a number of delivery systems have been tested in animal models. However, several viral vectors have been shown to trigger undesirable transient inflammatory responses in the eye. Previously, it was shown that an attenuated Herpes simplex virus vector (hrR3) transduced numerous cell types in the anterior and posterior segments of monkey eyes, but this was accompanied by inflammation. In the retina, retinal pigment epithelial cells were the predominant cell type transduced by hrR3. IL-6 is an important pro-inflammatory cytokine and may play a role in the response to the hrR3 vector. Infection of human ARPE-19 cells with hrR3 resulted in increased IL-6 expression and secretion 3 to 4 hours post-infection. In the presence of acyclovir (70 nM) or in cells infected with UV-inactivated hrR3, IL-6 was not up-regulated indicating viral replication was required. Expression of the HSV-1 α and β genes may be necessary but was not sufficient for NF- κ B activation and IL-6 up-regulation. The translocation of NF- κ B into the nucleus also occurred between 3 and 4 hours post-infection, coincident with increased IL-6 expression. Inhibition of NF- κ B translocation by an Adenovirus vector expressing a dominant negative I κ B (AdI κ Bam) inhibited IL-6 up-regulation, indicating that NF- κ B plays a role in increasing IL-6 expression in APRE-19 cells. The hrR3 virus lacks viral ribonucleotide reductase (RR) activity, thus RR is not required for NF- κ B activation or IL-6 up-regulation in ARPE-19 cells.

Keywords

ARPE-19 cells; Herpes Simplex Virus vector (hrR3); IL-6; ACV; NF- κ B

1. Introduction

The eye is an immunologically privileged organ where inflammatory responses are suppressed. Many factors are responsible for this suppression including the presence of the blood-aqueous barrier, high levels of transforming growth factor beta-2 (TGF β -2) (Jampel et al. 1990; Massague 1990; Streilein and Cousins 1990), complement inhibitors (Medawar 1948; Medof et al. 1987; Lass et al. 1990; Bora et al. 1993; Streilein 1993; Bardenstein et al. 1994; Streilein 1995) and the intraocular expression of Fas ligand (Griffith et al. 1995). However, ocular

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immune privilege can be compromised following infection with several pathogens. Herpes simplex virus (HSV) is a common human pathogen that infects epithelial cells. Corneal infection with HSV-1 typically causes epithelial cell damage and conjunctivitis. If the infection involves the deeper layers of the, scarring with loss of vision can occur. HSV also causes uveitis and acute retinal necrosis (Liesegang 1988; Kaufman et al. 1998; Liesegang 1999).

Ocular gene delivery has significant promise for dealing with various blinding diseases. Several viral-based vector systems including Adeno-associated virus (AAV), Adenovirus (AV), HSV, Lentiviral vectors (LV), and Baculoviruses (BV) have been used for ocular gene delivery in animal models or cell culture (Borras et al. 2002; Borras 2003; Liu et al. 2007a; 2007b). The majority of animal studies with viral vectors have used rodent models where gene delivery is well tolerated. In contrast, studies with rabbits have shown that ocular AV vector delivery triggers an inflammatory response (Borras et al. 1996). Non-human primates also develop ocular inflammation in response to AV, HSV, and LV even though the vectors do not replicate in the eye (Liu et al. 1999; Borras et al. 2002; unpublished data). These observations suggest that fundamental mechanistic differences in the induction of ocular inflammation exist between rodents and other species. The transient inflammatory responses in primate eyes can be reduced by lowering the dose of the vector but this reduces transduction efficiency. Although the inflammation is transient, this side effect must be eliminated if ocular gene therapy is to advance.

The HSV-ribonucleotide reductase null mutant hrR3 is a replication-competent avirulent Herpes Simplex Virus type 1 (HSV-1) carrying an insertion of the *lacZ* gene into the ICP6 (UL39) gene (Goldstein and Weller 1988). The ICP6 protein encodes the large subunit of the viral ribonucleotide reductase (RR) enzyme and hrR3 lacks functional RR. HSV-RR mutants are capable of establishing latency, but do not reactivate in rodents and are attenuated for virulence (Jacobson et al. 1989; Brandt et al. 1991; Idowu et al. 1992). They also do not cause acute necrotizing retinal disease when injected intravitreally in the rodent eye (Brandt et al. 1997; Spencer et al. 2000; Spencer et al. 2001).

We previously demonstrated that hrR3 could be used to deliver a foreign gene into rat, mouse, and monkey eyes (Liu et al. 1999; Spencer et al. 2000; Spencer et al. 2001). In the monkey study, transgene (*lacZ*) expression was detected in trabecular meshwork (TM) cells and in non-pigmented ciliary epithelial cells (NPE) following intracameral delivery. Expression of the *lacZ* transgene was predominantly detected in retinal-pigmented epithelial (RPE) cells. Sporadic retinal ganglion cells (RGC) were also transduced in eyes receiving virus intravitreally (Liu et al. 1999). A transient but significant inflammatory response in the anterior chamber, as well as mild vitritis and retinitis, was also observed in hrR3 transduced monkey eyes (Liu et al. 1999).

The pro-inflammatory cytokine Interleukin-6 (IL-6) is an important mediator of inflammation and has chemotactic activity for neutrophils and macrophages, activates T-lymphocytes, stimulates the secretion of immunoglobulin, and triggers the release of acute phase proteins (Planck et al. 1992; Feghali and Wright 1997). The local production of IL-6 by resident cells and infiltrating inflammatory cells has been detected in a variety of ocular inflammatory conditions (De Vos et al. 1992; Biswas et al. 2006). Studies have shown that cultured human RPE cells constitutively produce IL-6 and that either lipopolysaccharide (LPS) or IL-1 increases IL-6 production 2–5 fold (Chiba et al. 1993). Thus, the induction of IL-6 in RPE cells could be an early trigger of the retinal inflammatory response we observed following hrR3 delivery in primate eyes.

The IL-6 promoter contains consensus binding sites for the NF- κ B family of transcription factors and NF- κ B is required for the induction of IL-6 as well as other genes in several cell

types (www.nf-kb.org; (Rong et al. 1992; Matsusaka et al. 1993; Patel et al. 1998; Amici et al. 2001; Paludan 2001; Hargett et al. 2006; Melchjorsen et al. 2006). There are seven different NF- κ B family proteins that dimerize to form 15 possible complexes: p105, p50, p100, p52, p65, c-rel, and RelB. Antibodies to the p65 proteins are frequently used to monitor NF- κ B in cells (Carmody and Chen 2007). The NF- κ B proteins are normally sequestered in the cytoplasm through binding to I κ B proteins which suppresses expression of NF- κ B regulated genes including IL-6 (Tam et al. 2000). Upon the receipt of an inflammatory stimulus, the I κ B proteins are phosphorylated leading to polyubiquitination and proteosomal degradation (Delhase et al. 1999) and the release of NF- κ B to the nucleus. Given that NF- κ B controls IL-6 expression and that HSV-1 activates NF- κ B, it is possible that NF- κ B plays a role in IL-6 induction by HSV-1 in human RPE cells.

Our goals were to determine if IL-6 was induced following exposure of cultured human RPE cells to the hrR3 vector and whether induction required viral replication and NF- κ B activation. The results show that exposure of ARPE-19 cells to hrR3 increased IL-6 synthesis beginning 3–4 h post-infection. Induction was not detected when cells were exposed to UV-inactivated virus or when acyclovir was present, indicating that viral replication was required. Inhibitors of NF- κ B activation or expression of a dominant negative I κ B blocked the IL-6 induction, indicating that NF- κ B was involved.

2. Materials and Methods

2.1. Cell culture and hrR3 virus

ARPE-19 cells (Cat # CRL-2302, ATCC, Manassas, VA), were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium containing 1.2 g/L sodium bicarbonate, 2.5mM L-glutamine, 15mM HEPES, 0.5 mM sodium pyruvate (pH 7.2), 10% fetal bovine serum (FBS), 100 u/ml penicillin G, and 100 μ g/ml pstreptomycin sulfate at 37°C in 5% CO₂. The hrR3 vector contains an insertion of the *E. coli* β -galactosidase gene into the large subunit of HSV-1 (KOS) ribonucleotide reductase (UL39, ICP6; (Goldstein and Weller 1988)). Figure 1 shows a schematic representation of the structure of hrR3 genome. High titer viral stocks were prepared as described previously (Grau et al. 1989). Briefly, Vero cells were infected at a multiplicity of infection (MOI) of 0.01 to 0.1. When the CPE reached 90–100%, the cells and media were harvested, frozen and thawed 3 times, and centrifuged at 2,000 $\times$ g for 10 min to remove debris. The virus was then pelleted through a cushion of 36% sucrose in RSB buffer (10mM Tris, pH 7.4, 10mM NaCl, 3 mM MgCl₂) and resuspended in endotoxin free PBS (Visalli and Brandt 1993). The titers of the stocks were 1 $\times$ 10¹⁰ to 1 $\times$ 10¹¹ plaque forming units (pfu) per ml. Note that the hrR3 virus replicates in ARPE-19 cells but not in monkey or rodent tissue (Brandt et al. 1991; Spencer et al. 2000); unpublished data). To prepare UV-inactivated hrR3, a 10 cm dish containing 1 $\times$ 10⁷ hrR3 viral particles in 2.5 ml culture medium was exposed to the germicidal lamp (33 cm distance) in a class II biosafety cabinet for 1.5 h. Samples were exposed at ambient temperature. Inactivation was confirmed by assessing β -galactosidase expression as we described previously (Bultmann et al. 2001). Cell cultures were tested for mycoplasma using the MycoFluor™ Kit (Molecular Probes, Eugene, OR).

2.2. Construction of the recombinant I κ B α M adenoviral vector (AdI κ B α)

The plasmid pCMX-I κ B α M contains a dominant negative version of I κ B with mutations at S32 and S36 of the NH₂-terminus, a COOH-terminal PEST sequence mutation, and was kindly provided by Dr. Inder M. Verma (The Salk Institute, La Jolla, CA). The AdEasy Adenoviral system (Stratagene, La Jolla, CA) was used for vector construction. Briefly, the I κ B α M fragment was subcloned into pShuttle-CMV and the resultant pShuttle-I κ B α M plasmid was then used to generate adenoviral recombinants through homologous recombination with the

adenoviral backbone vector, pAdEasy-1, in BJ5183 bacterial cells (Stratagene, La Jolla, CA). After linearization with Pac I, the recombinant adenoviral vector DNA was transfected into HEK 293 cells to prepare vector stocks. A separate adenovirus vector expressing β -galactosidase (*lacZ*) alone (*AdlacZ*) was used as a control. Packaged virions were purified using a commercial kit (Cat # 70100, Puresyn, Inc., Malvern, PA). Viral titers were determined by optical density (particles per ml), and the recombinant vectors were then aliquotted and stored at -80°C . The titers of the *AdIkBaM* and *AdlacZ* vector stocks were 1.2×10^{12} and 3.76×10^{11} particles/ml respectively.

2.3. Analysis of IL-6

For immunoblotting, 7×10^6 ARPE-19 cells were infected with hrR3 at a multiplicity of infection (MOI) of 1. Following a 1 h absorption period, the unabsorbed virus was removed by rinsing the cells twice with PBS. The cells were then incubated in medium at 37°C . At 4, 8, and 12 h post-infection, the cells were washed twice with cold PBS containing protease inhibitor cocktail (Cat # P-8340, Sigma, St. Louis, MO), and collected in cold $1 \times$ PBS with protease inhibitor cocktail, and centrifuged at $500 \times g$ for 10 min. After removing the supernatant, 150 μl of Laemmli sample buffer (Cat # 161-0737, Bio-Rad, Hercules, CA) was added and the pellet was briefly sonicated. The protein concentration was measured with BCA Protein Assay Reagent (Cat # 23223, Pierce, Rockford, IL). After boiling for 5 min, equal amounts of protein were electrophoresed in a 12% denaturing polyacrylamide gel, and electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked for 1 h with 5% nonfat dry milk in Genius I buffer (100mM maleic acid, 150 mM NaCl pH 7.5) containing 0.3% v/v Tween-20 and then incubated with the desired primary antibody. For IL-6, polyclonal rabbit anti-IL6 IgG was used at a 1:200 dilution (Cat # SC-7290, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1.5 h at room temperature. After washing, the filters were incubated for 1.5 h at room temperature with goat anti-rabbit IgG-conjugated alkaline phosphatase at a 1:1000 dilution (Cat # A-3687, Sigma) and washed. The immunoblots were developed either with 5-bromo-4-chloro-3-indolyl phosphate nitroblue tetrazolium substrate (Cat # B5655, Sigma Aldrich, St. Louis, MO) or the enhanced chemiluminescence (ECL) system (Amersham Biosciences, Piscataway, NJ). Immunoblots were quantified using Scion Image 4.0.3.2 Software (Scion Corp., Frederick, MD).

The secretion of IL-6 was quantified using a commercial ELISA kit (Cat # 850.030.096, Cell Sciences Inc., Canton, MA) according to the instructions from the manufacturer. The lower limit of detection for IL-6 was 2 pg/ml. The results were quantified by reading the absorbance at 450 nm with an ELISA plate reader (Spectra MAX 250, Molecular Devices Corp., Sunnyvale, CA). Significant differences were determined using the paired t-test.

2.4. Analysis of IkBaM

For immunoblotting, ARPE-19 cells were transduced with different amounts of adenovirus vector. At 2 days post-transduction, lysates were prepared and immunoblotting was performed as described above. Mouse monoclonal anti- IkBa (H4) antibody (SC-1643, Santa Cruz, Santa Cruz, CA) and rabbit anti-mouse IgG conjugated with horseradish peroxidase (HRP) (SC-2031, Santa Cruz, Santa Cruz, CA) were used as the primary and secondary antibodies respectively. Immunoreactive bands were detected using the enhanced chemiluminescence (ECL) system (Amersham Biosciences, Piscataway, NJ). To confirm equal loading of the lanes, the blot was washed once with $1 \times$ PBS, incubated with stripping buffer (0.2 M Glycine-HCL, pH 2.5, 0.05% Tween-20) at room temperature for 1 h, then rinsed with PBS. The blot was then blocked and re-probed with anti-goat actin polyclonal antibody (SC-1616, Santa Cruz, Santa Cruz, CA) followed by secondary bovine anti-goat IgG-HRP (SC-2350, Santa Cruz, Santa Cruz, CA).

For immunofluorescence, ARPE-19 cells were seeded in 4-well chamber slides at 50% confluence (Cat # 154526, Nalge Nunc International, Naperville, IL). Twenty-four hours later the cells were exposed to hrR3 or UV inactivated hrR3 at a MOI of 2. After a 1 h incubation at 37°C, the cells were washed twice with PBS and then incubated in medium at 37°C. At the desired time after virus exposure, the cells were washed three times with 0.05% BSA/PBS, fixed with 4% paraformaldehyde in PBS for 1 h at room temperature, and permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature with gentle agitation. The cells were then washed and blocked by incubating them in 5% normal donkey serum (Cat # 017-000-121, Jackson Immuno Research Laboratories, West Grove, PA) in 0.1 M TBS for 1 h. The cells were then incubated with polyclonal goat anti-human NF- κ B p65 antibody (Cat # SC-372 G, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:50 in 2.5% normal donkey serum for 1 h at room temperature. Cells were then washed three times with 0.1 M TBS and reacted with Cy 3-conjugated AffiniPure Donkey Anti-Goat IgG (H+L) (Cat # 705-165-147, Jackson Immuno Research Laboratories, West Grove, PA) at a 1:100 dilution in 2.5% normal donkey serum for 1 h at room temperature. The cells were then stained with Hoechst 33342 (Cat # H21492, Molecular Probes, Inc., Eugene, OR) at concentration of 1 μ g/ml in 0.1 M TBS for 5 min, washed twice with 0.1 M TBS, and rinsed once with distilled water. The slides were mounted with GEL/MOUNT (Cat # M01, Biomedica Corp., Foster City, CA) and fluorescence was observed with a Zeiss Axioplan 2 microscope equipped with an Axiocam HRm camera together with Axiovision 3.1 software (Carl Zeiss Microimaging Inc., Thornwood, NY).

2.5. Transduction with AdIkBa or AdlacZ

The ARPE-19 cells were grown to confluence in the culture medium containing 10% FBS. The cells were then transduced with the AdIkBa or AdlacZ at a MOI of 120. Following a 2 h absorption period, unabsorbed virus was removed by rinsing the cells twice with PBS, and the cells were incubated in medium at 37°C.

2.6. Analysis of NF- κ B activation

The ARPE-19 cells were seeded in 4-well chamber slides (Cat # 154526, Nalge Nunc International, Naperville, IL). Twenty-four hours later the cells were transduced with AdIkBa or AdlacZ at MOI of 120. After a 2 h incubation period at 37°C, the cells were washed twice with PBS and then incubated at 37°C. At 24 hours after transduction, the cells were treated with IL-1 β (100 ng/ml) for 1 h or hrR3 at a MOI of 2 for 8 h. After treatment, the cells were washed and NF- κ B p65 was detected by immunofluorescence as described above.

2.7. In vitro growth curves

Monolayers of RPE cells in six-well tissue culture plates were infected with hrR3 at a MOI of 4 and incubated for 1 h at 37°C. The cells were then washed twice with PBS and were then incubated in complete growth medium at 37°C. Infected cells were scraped from the wells at various times post-infection and centrifuged at 3,000 $\times$ g. The supernatant was saved, and the cells were frozen and thawed three times in dry ice/ethanol to release the virus. Cellular debris was removed by centrifugation for 10 min at 2,000 $\times$ g and the resulting supernatant was combined with the culture medium. The total amount of virus per sample was then determined by standard plaque assay in triplicate on Vero cell monolayers.

2.8. Acyclovir (ACV) inhibition assay

The ARPE-19 cells were infected with hrR3 at a MOI of 2 in the presence of 70 nM acyclovir (Cat # 59277-89-3, Sigma, St. Louis, MO) to prevent viral DNA replication. At 3, 8, and 12 h post-infection, culture supernatants were collected and IL-6 was quantified in triplicate by ELISA as described above.

3. Results

3.1. Induction of IL-6 in ARPE-19 cells infected with hrR3

To determine if IL-6 was induced by hrR3, ARPE-19 cells were infected with hrR3 at a MOI of 1 and IL-6 expression was analyzed by immunoblotting using an anti-IL-6 antibody. Two major bands at approximately 40kDa and 28kDa were detected in hrR3-infected but not mock-infected cells in accordance with other studies showing that IL-6 is present in multiple forms in cells (May et al. 1989). Figure 2A shows that IL-6 expression was first detected at 4 h post-vector exposure and continued out to 12 h. In this particular blot, constitutive expression of IL-6 occurred at low levels. In other experiments (see below) levels of constitutive expression were higher. The reason for the variability in constitutive IL-6 expression in ARPE-19 cells is not presently known. The cells were not contaminated with mycoplasma and serum endotoxin levels were 10 Eu/ml. To determine if IL-6 secretion was increased in cells infected with hrR3, culture medium was collected and assayed for IL-6 using a commercially available ELISA kit. As shown in Figure 2B, cultured ARPE-19 cells constitutively secreted IL-6, as has been reported previously (Chiba et al. 1993). When compared to control cultures, significantly increased secretion of IL-6 was seen at 8 (p <0.05) and 12 h (p = <0.05) post-infection in cells exposed to hrR3. Increased IL-6 RNA was detected at 3 h post-infection by RT-PCR but this was not quantified (data not shown). These data indicate IL-6 production was increased in ARPE-19 cells infected with hrR3 and that induction occurred between 3 and 4 hours after post-infection.

3.2. hrR3 induces NF- κ B activation

The IL-6 promoter contains binding sites for nuclear factor κ B (NF- κ B) and the requirement of NF- κ B activation for IL-6 induction has been well documented (Rong et al. 1992; Matsusaka et al. 1993; Patel et al. 1998; Amici et al. 2001; Paludan 2001; Hargrett et al. 2006; Melchjorsen et al. 2006). We therefore asked if NF- κ B was activated in hrR3 infected ARPE-19 cells by measuring translocation of NF- κ B from the cytoplasm to the nucleus. Figure 3 shows representative examples of control and hrR3-treated cells at 30 min (3A) and 8h (3B) post-infection. A quantitative analysis of the data showed that nuclear translocation of NF- κ B was first detected at 4 h post-infection with hrR3 (Figure 3C). By 6 h post-infection, 51.9% of the nuclei were positive for NF- κ B compared to 7.1% in the controls. By 8 h, 93.3% of the hrR3 nuclei were positive, compared to 3.2% in control cells. As a positive control, we also included IL-1 β , which is a known activator of NF- κ B (DiDonato et al. 1996). Treatment of ARPE-19 cells with IL-1 β rapidly induced NF- κ B nuclear translocation with 93.6% of cells positive at 30 min post-treatment (Figure 3C). There was no difference between the mock-only treated control and hrR3 infected cells at the 30 min time point. These results indicated that infection with hrR3 induces NF- κ B translocation and that the timing of the translocation appeared to be co-incident with the IL-6 induction that occurred between 3 and 4 h post-infection.

3.3. Inhibition of NF- κ B activation blocks IL-6 induction

To confirm that NF- κ B activation was involved in the hrR3-induced IL-6 up-regulation, we generated an adenovirus vector expressing a dominant negative mutant of I κ BaM. To confirm the expression of the I κ BaM from the AdI κ BaM vector, cell lysates from transduced ARPE-19 cells were immunoblotted and probed with a polyclonal antibody specific for I κ Ba. As shown in Figure 4A, dose-dependent expression of a 32kDa protein corresponding to I κ BaM was seen in AdI κ BaM-transduced ARPE-19 cells but not in mock-transduced cells. Lower molecular weight bands in the AdI κ BaM-transduced lanes may be degradation products of the I κ BaM protein. The higher molecular weight bands present in some of the blots with transduced samples are most likely post-translationally modified forms of I κ Ba. Probing the blots for actin confirmed the lanes were equally loaded (Figure 4A).

We next asked if the dominant negative I κ BaM blocked IL-1 β induced NF- κ B nuclear translocation in ARPE-19 cells. Cells were first transduced with AdI κ BaM or AdlacZ for 24 h and the cells were exposed to IL-1 β for 1 h. Immunostaining for p65 showed that a strong nuclear staining was observed in IL-1 β stimulated AdlacZ transduced cells (Figure 4B). In AdI κ BaM transduced cells, NF- κ B p65 was located exclusively in the cytoplasm (Figure 4B). The amount of AdI κ BaM required to completely block IL-1 β induced NF- κ B nuclear translocation was 120 transducing units/per cell. At this dose, no cellular morphology change was observed under light microscopy (data not shown).

Having confirmed that the AdI κ BaM vector blocked the IL-1 β induced NF- κ B activation, we then asked whether AdI κ BaM inhibited hrR3 mediated NF- κ B nuclear translocation. Cells were pretreated with AdI κ BaM or AdlacZ for 24 h. The cells were then infected with hrR3 at a MOI of 2 for 8 h and p65 localization was assessed by immunofluorescence. Strong nuclear staining was observed after hrR3 infection in AdlacZ- transduced cells whereas in AdI κ BaM-transduced cells, NF- κ B p65 was localized exclusively in the cytoplasm (Figure 4C). These results indicated that the AdI κ BaM inhibited hrR3 induced NF- κ B nuclear translocation. We then asked if the induction of IL-6 by hrR3 could be blocked by AdI κ BaM. ARPE-19 cells transduced by AdlacZ or AdI κ BaM were infected with hrR3 at a MOI of 2 for 8 h and IL-6 expression was analyzed by western blotting. As shown in Figure 4D, hrR3 induction of IL-6 was reduced in cells expressing I κ BaM but not in AdlacZ transduced cells suggesting that IL-6 induction by hrR3 requires NF- κ B activation.

3.4. Viral replication is required for IL-6 induction

The hrR3 virus is capable of replicating in some cultured cell lines, but not in rodent eyes (Brandt et al. 1991; Brandt et al. 1997; Spencer et al. 2000). It was, therefore, of interest to determine if hrR3 replicated in ARPE-19 cells. As shown in Figure 5A, we found that the hrR3 virus showed a characteristic one step growth curve with an eclipse period lasting until 6–9 hours post-infection. Increased viral titers were seen at 9 hours post-infection and continued to increase up to 24 hours indicating hrR3 replicated in ARPE-19 cells.

To determine if viral replication was required for the induction of IL-6, we infected ARPE-19 cells with hrR3 in the presence of 70 nM acyclovir and assayed the culture medium for IL-6 by ELISA. The results plotted as fold-differences over the control cultures are shown in Figure 5B. There was little difference in IL-6 expression between hrR3 and hrR3 plus ACV-treated samples versus control samples at 3 h post-infection ($p>0.05$). At 8 h post-infection, there was an approximately 2-fold increase in secreted IL-6 in the hrR3 treated cells versus control cells, but not in hrR3 plus ACV-treated cells ($p<0.05$). The difference increased to 3-fold by 12 h post-exposure to hrR3 ($p<0.05$). The actual IL-6 concentrations (pg/ml/ 10^5 cells) at 8h were 73.0 ± 17 , 148.0 ± 32 , and 74.1 ± 16 for the control, hrR3 only, and hrR3 + ACV respectively. At 12 h the IL-6 concentrations were 66.0 ± 15 , 198.8 ± 44 , and 63.0 ± 12 pg/ml/ 10^5 cells respectively for the control, hrR3 only, and hrR3 + ACV cultures. These data indicate that hrR3 replication is required for the induction of IL-6 in ARPE-19 cells. To confirm that viral replication was required for hrR3-induced IL-6 expression, we asked if UV-inactivated hrR3 was able to induce IL-6. When ARPE-19 cells were exposed to UV-inactivated hrR3, immunostaining showed that NF- κ B nuclear translocation was induced (Figure 5C). Immunoblotting showed that at 8 h post-hrR3 infection there was a 1.6-fold increase in IL-6 compared to the controls whereas there was no increase in cells exposed to UV-inactivated virus (Figure 5D). These results confirmed that viral replication was required for NF- κ B activation and IL-6 induction in ARPE-19 cells.

4. Discussion

The injection of HSV, Ad, or LV vectors into the anterior chamber or vitreous of non-human primates triggers a transient inflammatory response (Liu et al. 1999; Borrás et al. 2001; unpublished data). In contrast, injection of HSV vectors into rodent eyes does not trigger inflammation (Brandt et al. 1997; Spencer et al. 2000; Spencer et al. 2001), indicating that fundamental differences exist between rodent and non-human primates regarding inflammatory triggers. The goal of this work was to test the potential involvement of the pro-inflammatory cytokine IL-6 in triggering ocular inflammation by the attenuated hrR3 HSV vector in primates. We focused on RPE cells since they are one of two principle cell types transduced by this vector in the primate retina (Liu et al. 1999). Our results showed that exposure of ARPE-19 cells to hrR3 resulted in increased IL-6 expression beginning 3 to 4 h post-infection. The increased IL-6 expression required viral replication, as acyclovir blocked the increase and UV inactivated virus did not induce IL-6. In addition, the increase in IL-6 was dependent on the activation and nuclear translocation of NF- κ B.

Increased expression of IL-6 was not seen until 3–4 h post-exposure of ARPE-19 cells to hrR3 and the timing of the induction coincided with translocation of NF- κ B to the nucleus, suggesting that activation of NF- κ B was required for IL-6 induction. This is consistent with findings in other cell types showing a role for NF- κ B in regulating IL-6 expression (Matsusaka et al. 1993). The delayed IL-6 induction and NF- κ B activation by hrR3 contrasts with IL-1 β , where NF- κ B nuclear translocation peaked 30 minutes after exposure. These results suggest that hrR3 and IL-1 β use different pathways to activate NF- κ B.

The activation of NF- κ B at 3–4 h post-infection in ARPE-19 cells is similar to the timing of NF- κ B activation in other cell types infected with HSV (Patel et al. 1998; Amici et al. 2001; Goodkin et al. 2003; Taddeo et al. 2003; Amici et al. 2006). A transient activation of NF- κ B that occurs immediately upon infection has also been reported to occur in a human keratinocyte cell line (HaCaT) (Amici et al. 2006). This early activation occurs with UV-inactivated virus, suggesting that either viral attachment or entry is the trigger. In addition, viral attachment to U937 cells induces NF- κ B activation after a lag of 3 h and this is due to gD binding to HveA (Teresa Sciortino et al. 2007). Our observation that UV-inactivated hrR3 virus did not induce NF- κ B translocation or IL-6 induction at 30 min post-exposure suggests that this early pathway is not functional in ARPE-19 cells. However, more detailed studies will be required to confirm this. Our findings are also consistent with several reports showing HSV induces a persistent activation of NF- κ B in several cell types (Rong et al. 1992; Patel et al. 1998; Amici et al. 2001; Taddeo et al. 2003; Gregory et al. 2004; Hargett et al. 2006).

Several studies have suggested that HSV immediate early and/or early proteins are required for the persistent NF- κ B activation and induction of IL-6. In C33 cells, infection by HSV-1 with mutations in ICP-4 or ICP-27 did not activate NF- κ B (Patel et al. 1998). In macrophages, where NF- κ B activation was required for induction of RANTES, deletion of the ICP-0 gene prevented induction whereas deletion of ICP-4, ICP-27, and ICP-22 had no effect (Melchjorsen et al. 2002; Melchjorsen and Paludan 2003). In contrast, ICP-27 appears to be required for NF- κ B activation in Hep-2 and CV-1 cells (Goodkin et al. 2003; Hargett et al. 2006). These results indicate that mechanism of activation of NF- κ B depends on the cell type. The observation that acyclovir blocked IL-6 induction in ARPE-19 cells suggests that expression of the HSV α and β genes may be necessary, but is not sufficient for IL-6 induction and that some other event is required (Melchjorsen et al. 2006).

HSV expresses a distinct ribonucleotide reductase (RR) that consists of two heterogeneous protein subunits (Anderson et al. 1981; Dutia 1983; McLauchlan and Clements 1983; Bacchetti et al. 1984; Cohen et al. 1985; Frame et al. 1985; Bacchetti et al. 1986; Ingemarson and

Lankinen 1987). Our data suggest that the HSV-1 RR activity is not needed for IL-6 induction and hrR3 replication in APRE-19 cells since the *lacZ* insertion in hrR3 disrupts the ICP-6 gene (Goldstein and Weller 1988).

The activation of NF- κ B and induction of IL-6 at 3–4 h after exposure of the ARPE-19 cells coincides with the onset of expression of HSV-1 β genes and viral DNA replication. The fact that acyclovir, which blocks viral DNA replication but does not inhibit α or β gene expression, inhibits IL-6 induction indicates that viral DNA replication *per se* or some event requiring the onset of viral DNA replication is involved in the induction of IL-6. The presence of unmethylated CpG motifs in HSV DNA is recognized by TLR-9 (Lund et al. 2003), which can activate NF- κ B, thus the TLR-9 pathway may be involved in IL-6 induction by hrR3. Infection of corneal cells with HSV-1 induces TLR-9 gene expression and the TLR-9 inhibitor iODM blocks IL-6 induction in these cells (Hayashi et al. 2006). Studies are underway to determine whether TLR-9 is involved in the IL-6 induction in ARPE-19 cells.

The activation of NF- κ B also depends on the activation of protein kinase R (PKR), which phosphorylates IKK (Zamanian-Daryoush et al. 2000). The activation of PKR may be involved in the activation of NF- κ B in HSV-infected cells (Taddeo et al. 2003). Additional evidence for the involvement of PKR in NF- κ B activation comes from the observation that expression early in infection of the HSV-1 U_s11 protein which blocks PKR, prevents NF- κ B activation (Taddeo et al. 2003). Whether PKR is involved in NF- κ B activation and IL-6 induction ARPE-19 cells will require additional studies.

In summary, we report several new findings regarding the induction of IL-6 by HSV-1 in human retinal pigment epithelial cells. Infection of ARPE-19 cells results in the activation of NF- κ B and IL-6. We have also shown that IL-6 induction requires both the replication of HSV-1 and NF- κ B activation. Our data also suggest that the expression of viral α and β genes may be necessary but is not sufficient for NF- κ B activation and IL-6 induction and the viral ribonucleotide reductase enzymatic activity is not required for NF- κ B activation and IL-6 induction.

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Figure 1. The structure of hrR3 genome

The top line shows the structure of the HSV-1 genome. The second line shows an enlargement of the region encoding the large subunit of ribonucleotide reductase (UL 39 gene; nucleotides 86,644 to 89,857) and the insertion of the *lacZ* gene in the UL39 locus at position 87,816 in the HSV-1 genome. TRL, terminal repeat long; UL, unique long; IRL, internal repeat long; IRS, internal repeat short; US, unique short; TRS, terminal repeat short; hrR3, herpes simplex virus type 1 ribonucleotide reductase mutant; *lacZ*, *lacZ* gene.

Figure 2. IL-6 expression in hrR3 infected ARPE-19 cells

Panel A. Immunoblotting of cell extracts. The ARPE-19 cells were grown to confluence in culture medium containing 10% FBS, and the cells were infected with hrR3 at a multiplicity of infection (MOI) of 1. Cell cultures were harvested at 4, 8 and 12 h post-infection. Cell lysates were electrophoresed and subjected to western blotting with polyclonal rabbit anti-human IL-6 antibody. The positions of protein molecular weight markers are shown on the right and IL-6 isoforms are indicated. This blot is representative of three separate experiments showing IL-6 is up-regulated in infected cells. **Panel B. Effect of hrR3 infection on IL-6 secretion.** ARPE-19 cell cultures grown to confluence were either mock-infected or infected with hrR3 at a MOI of 1. The culture supernatants were harvested at 3, 8, and 12 h post-infection and analyzed for IL-6 by ELISA. Cell numbers were counted at each time point with a hemacytometer. Results are presented as the mean $\pm$ standard error of the mean for three independent experiments. Filled bar: control. Shaded bar: hrR3.

Figure 3. hrR3 induced NF- κ B p65 nuclear translocation in ARPE-19 cells at 30 min or 8 h after treatment

ARPE-19 cells, seeded in 4-well chamber slides, were infected with hrR3 at a MOI of 2, IL-1 β (100 ng/ml), or with medium as a control. Thirty minutes (Panel A) or 8 h (Panel B) following the treatments the cells were immunostained with antisera specific for the p65 subunit of NF- κ B. The nuclei were also stained with Hoechst dye and Hoechst images were pseudocolored green to facilitate image merger. All images were originally taken at 100x magnification. Panel C. The percentages of cells with nuclear NF- κ B p65 at various time points post-treatment. □, IL-1 β ; Δ, hrR3; ○, IL-1 β control; ∇, hrR3 control.

Figure 4. (A) Immunoblot analysis of IkBaM expression in AdIkBaM transduced ARPE-19 cells The ARPE-19 cells were grown to confluence and either mock-transduced or transduced with AdIkBaM at a MOI of 5 or 50. Cell lysates were then subjected to western blotting using mouse polyclonal anti-IkBa antibody. Actin staining was used to show the lanes were loaded equally. **Panels B and C.** IL-1 beta (B) or hrR3 (C) induced NF-κB p65 nuclear translocation in AdIkBaM or AdLacZ transduced ARPE-19 cells. ARPE cells were seeded in 4-well chamber slides. The following day the cells were transduced with the AdIkBaM or AdLacZ viruses for 24 h. The cells were then treated with IL-1 β for 1 h or hrR3 for 8 h. Following treatments, the cells were immunostained with antiserum specific for p65. Hoescht stained images were pseudocolored green to facilitate merging of the images. All images were originally taken at a magnification of 40x. **(D). Immunoblot analysis of hrR3 induced IL-6 expression in AdIkBaM or AdLacZ transduced ARPE-19 cells.** The ARPE-19 cells were grown to confluence and then transduced with AdIkBaM or AdLacZ. Twenty-four hours later the cells were then infected with hrR3 at a MOI of 1. At 8 h post-infection, cell lysates were subjected to western blotting with polyclonal rabbit anti-human anti IL-6. The blot was then stripped and re-probed with actin antibody as a loading control. Signals were detected using the ECL system. The positions of IL-6 isoforms are denoted with arrows.

Figure 5. Viral replication is required for IL-6 induction and NF-κB activation

(A). One-step growth curve of hrR3 and KOS in ARPE-19 cells. ARPE-19 cell cultures were infected with hrR3 or HSV-1 KOS at an MOI of 4. After a 1 h adsorption period, the monolayers were washed three times and refed. At the indicated times, the cultures were collected and the amount of total virus per sample was titered by plaque assay using Vero cells. Each data point represents the mean $\pm$ standard error of the mean titer from three independent experiments. $\circ$, HSV-1 KOS; $\bullet$, hrR3. **(B). IL-6 secretion in hrR3 or hrR3 plus ACV-infected ARPE-19 cells.** Cell cultures were infected with hrR3 in the presence or absence of 70 nM ACV. After a 1 h adsorption period, the monolayers were washed three times and replenished with culture medium or culture medium with ACV. At the indicated times, culture supernatants were harvested, and the IL-6 concentration was determined by ELISA. The fold induction of IL-6 between hrR3 or hrR3 plus ACV-treated samples vs. control samples was

calculated for each time point. The points represent the means $\pm$ standard error of the means for triplicate samples. ■, hrR3 + ACV; ■, hrR3. **(C). hrR3 or UV-inactivated hrR3 induction of NF- κ B p65 nuclear translocation in ARPE-19 cells.** ARPE-19 cells were seeded in 4-well chamber slides the day before experiment. On the day of experiment, cells were infected with hrR3 or UV-inactivated hrR3 and eight hours later they were immunostained for NF- κ B p65. Hoescht stained images were pseudocolored green to facilitate image merger. All images were originally taken at a magnification of 40x. **(D). Immunoblot analysis of hrR3 or UV-inactivated hrR3 induced IL-6 expression in ARPE-19 cells.** The ARPE-19 cells were grown to confluence and the cells were infected with hrR3 or UV-inactivated hrR3 at a MOI of 1 (the titer for UV-inactivated hrR3 was the titer prior to UV-irradiation). At 8 h post-infection cell lysates were subjected to western blotting with polyclonal rabbit anti-human IL-6. The blot was the stripped and re-probed with actin antibody as a loading control. Signals were detected using ECL system and the signal intensities were determined using Scion Image 4.0.3.2 (Scion Corp., Frederick, MD).